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PURIFICATION AND CHARACTERIZATION OF HEXOSE-OXIDASE FROM THE RED ALGA, CHONDRUS CRISPUS

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PURIFICATION AND CHARACTERIZATION OF HEXOSE OXIDASE FROM THE RED ALGA *CHONDRUS CRISPUS*

by

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ABSTRACT

PURIFICATION AND CHARACTERIZATION
OF HEXOSE OXIDASE FROM THE RED ALGA CHONDRUS CRISPUS

by

James D. Sullivan, Jr.

Hexose oxidase (D-hexose:O_2 oxidoreductase, EC 1.1.3.5) has been isolated, purified, and characterized from the red alga, Chondrus crispus. The enzyme oxidizes the following substrates: D-glucose, D-galactose, maltose, lactose, and cellobiose. Products of the reaction include hydrogen peroxide and the sugar lactone. The production of hydrogen peroxide has been shown responsible for the growth-inhibitory effect of C. crispus to Chlorella pyrenoidosa. Optimum temperature and pH for the Chondrus hexose oxidase reaction are 25°C and 6.3, respectively. A molecular weight of approximately 130,000 has been determined by gel filtration on Sephadex G-200. The purified enzyme contains ca. 0.6% copper which represents about 12 gram atoms Cu per mole of enzyme of molecular weight 130,000. Chondrus hexose oxidase is a glycoprotein containing ca. 70% carbohydrate which consists mainly of galactose and xylose. Flavin adenine dinucleotide, the coenzyme of glucose oxidase, is not detectable in the Chondrus enzyme. Resistance to proteolytic digestion with pepsin and trypsin is found. Approximately 11% of the

original activity is recoverable following a purification procedure involving n-butanol treatment, ammonium sulfate precipitation, DEAE-cellulose chromatography, pepsin-trypsin digestion, and gel filtration on Sephadex G-200. The purified enzyme shows a single band staining with Coomassie blue on disc gel electrophoresis at pH 8.0.

INTRODUCTION

The unicellular green alga Chlorella pyrenoidosa has been shown to be a particularly useful organism for assaying various toxins of fungal and algal origin (1). In most cases, toxin-containing paper disks or toxin-producing organisms when placed on a Chlorella-seeded agar plate produce a circular zone of inhibition which appears colorless against a green background. By this method, numerous compounds at a given concentration or organisms themselves can easily be screened for toxicity. Not all species of Chlorella however are equally sensitive when certain compounds are screened (2). The growth-inhibitory activity of an extensive list of compounds against several Chlorella strains is found in the APPENDIX of this thesis. C. pyrenoidosa (UNH strain) has been used almost exclusively in this investigation.

Two red algae, Chondrus crispus and Euthora cristata, have been shown to inhibit the growth of C. pyrenoidosa (1). The objective of the study reported herein has been the isolation, purification and characterization of the causative substance in Chondrus. Some preliminary studies have been done with E. cristata although not as detailed due to the limited quantity available.

The compound which appears to be directly involved in the growth-inhibitory response is hydrogen peroxide.

The production of H_2O_2 has been attributed to the action of a "glucose oxidase" in C. crispus on glucose which is a constituent of the Chlorella growth medium. A procedure for purifying this enzyme has been developed and information on its properties gathered.

An enzyme, referred to as carbohydrate oxidase and quite similar in properties to the Chondrus enzyme, has been isolated and partially purified from the red alga Iridophycus flaccidum (3). This type of enzyme has since been named D-hexose:O_2 oxidoreductase (EC 1.1.3.5) or simply hexose oxidase because a somewhat unusual property of the Iridophycus enzyme is its wide range of substrate specificity which includes D-glucose, D-galactose, maltose, lactose, and cellobiose. This characteristic distinguishes this enzyme from glucose oxidase (EC 1.1.3.4) which is highly specific for D-glucose. In addition to being found in some red algae, "glucose oxidases" are also known to occur in honey (4), bacteria (5,6), fungi (7-10), and citrus fruits (11). Most of these enzymes could be expected to inhibit the growth of C. pyrenoidosa through their action on D-glucose which results in H_2O_2 production.

The bacterium Malleomyces pseudomallei has been reported to contain an enzyme with nearly equal specificity for D-glucose and D-galactose (5). To what extent, if any, maltose, lactose, and cellobiose are attacked, has not been shown. If these disaccharides are oxidized, it is quite possible the bacterial enzyme closely resembles

the Iridophycus enzyme in other properties as well. The oxidation product of D-glucose in the presence of this enzyme is D-gluconic acid with the lactone occurring as an intermediate in the reaction. The formation of the aldonic acid (or lactone) appears to be quite typical of "glucose oxidases".

Species of Aspergillus and Penicillium are known to contain glucose oxidase which has been characterized as a flavin-containing enzyme with a molecular weight between 150,000 and 160,000. The main characteristic of this enzyme is the presence of flavin adenine dinucleotide (FAD). Another enzyme containing FAD is lactose dehydrogenase which oxidizes in addition to lactose: D-glucose, D-galactose, D-mannose, L-arabinose, D-ribose, D-xylose, and maltose. Lactobionolactone is the product of the enzymatic oxidation of lactose (6). In several other partially purified "glucose oxidases" FAD has not been detected (3-5, 9, 11).

A coenzyme other than FAD has been found in an enzyme bearing some relation to glucose oxidase. This particular enzyme is galactose oxidase which contains 1 gram atom copper per mole of enzyme of molecular weight 75,000 (12). Although H_2O_2 is produced in the reaction, the oxidation of D-galactose occurs at the C-6 position giving rise to a hexodialdose (13) rather than C-1 oxidation, which in the case of glucose oxidase results in the formation of the aldonic acid (or lactone).

Purified galactose oxidase does not oxidize D-glucose at a detectable rate (13). Glucose oxidase and galactose oxidase are highly substrate specific, produce H_2O_2, and contain as coenzyme FAD and copper, respectively. Copper has not previously been reported as a constituent of "glucose oxidases".

C. pyrenoidosa appears to be a good assay organism against which numerous algae could be screened for "glucose oxidase" activity. Although not all inhibition may be due to the action of such an enzyme, the possibility of H_2O_2 as the growth-inhibitory substance is quite good. Whether or not H_2O_2 is responsible can be determined by assaying an aqueous algal extract with the o-dianisidine-peroxidase system described in the METHODS section. In the presence of H_2O_2 and peroxidase, the chromagen, o-dianisidine is transformed to a colored product. If this conversion does not result, something other than H_2O_2 may be involved in the growth-inhibitory response.

MATERIALS AND METHODS

The following were obtained from commercial sources: Sephadex G-200 (Pharmacia Fine Chemicals), Whatman DE 52 DEAE-cellulose (Reeve-Angel), pepsin and trypsin (Nutritional Biochemicals); Aspergillus niger glucose oxidase (1100 units/ml), o-dianisidine diHCl, and peroxidase (Sigma Chemical). Standards for gel filtration included ribonuclease (Nutritional Biochemicals), and myoglobin, chymotrypsinogen, ovalbumin, albumin, gamma globulin, apoferritin (Schwarz-Mann). Other chemicals used were of reagent grade.

Determination of Protein and Carbohydrate

Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard and carbohydrate by the anthrone method (15) using D-galactose as the standard. The standard curves are depicted in Figs. 1 and 2, respectively.

Copper Determination

Copper was determined by atomic absorption spectroscopy and the dithizone method (16). Before either determination was made, the lyophilized sample (ca. 10 mg) was wet-ashed with a 3.5 ml mixture containing 3 ml nitric acid and 0.5 ml 35% perchloric acid and then neutralized with ammonium hydroxide (16). With the dithizone method, a standard curve was obtained using $CuSO_4 \cdot 5H_2O$ as the source of copper (Fig. 3).

Fig. 1. Standard curve for determination of protein by Lowry method using bovine serum albumin.

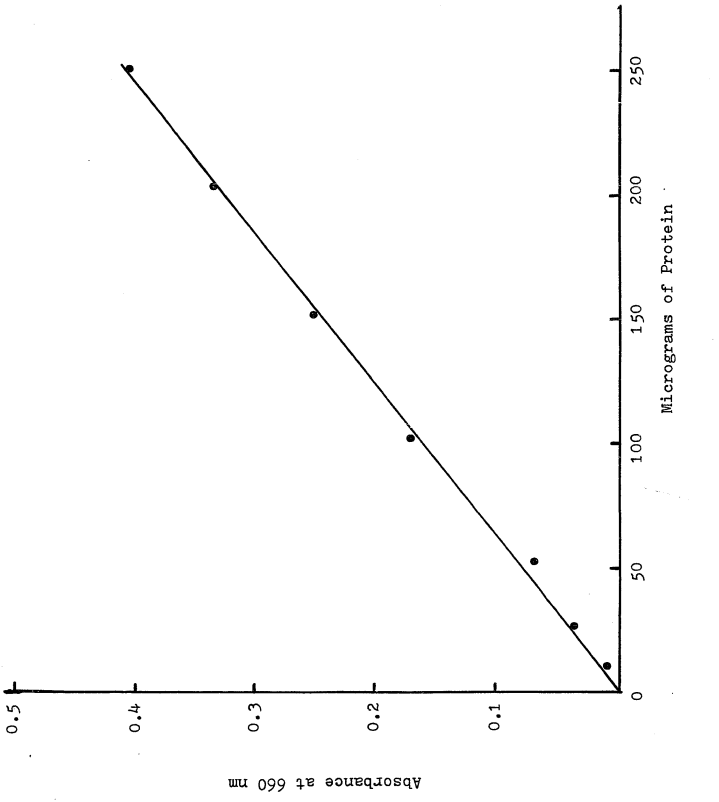


Fig. 2. Standard curve for determination of carbohydrate by anthrone method using D-galactose.

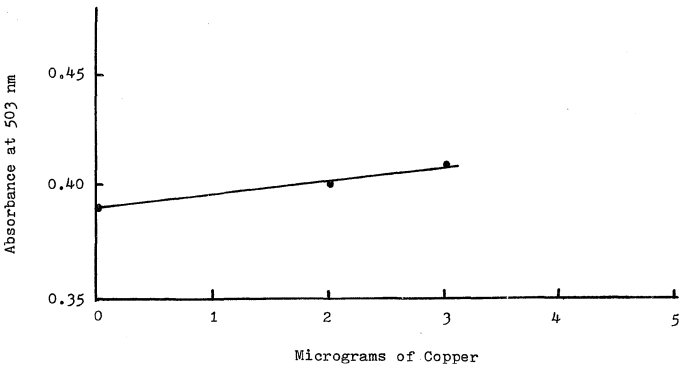


Fig. 3. Standard curve for determination of copper using dithizone method.

Disc Gel Electrophoresis

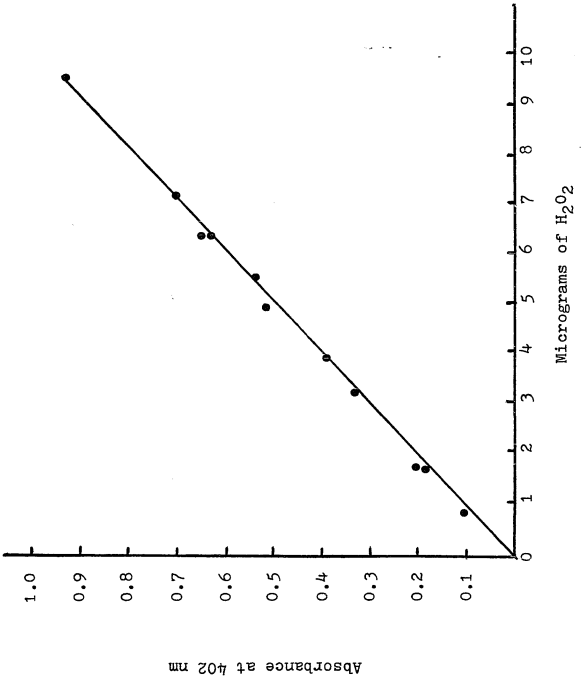
The purity of Chondrus hexose oxidase was determined by disc gel electrophoresis. Standard gels (7%) were run at 5°C and 2 mA per tube, using a Tris-barbital buffer with a running pH of 8.0 as described by Williams and Reisfeld (17). Gels were stained with Coomassie blue (0.25% in methanol:water:acetic acid, 5:5:1) and destained electrophoretically with a Canalco gel destainer using 7% acetic acid.

Assay of Hexose Oxidase

o-dianisidine-peroxidase system:

The procedure used for assaying the Chondrus enzyme was based on methods given for the assay of glucose oxidase (4,18). The assay mixture consisted of the following: 1.5 ml glucose (0.1 M in 0.1 M sodium phosphate pH 6.3), 1.2 ml sodium phosphate buffer pH 6.3, 0.1 ml o-dianisidine diHCl (3.0 mg/ml in water), 0.1 ml peroxidase (0.1 mg/ml in sodium phosphate buffer), and 0.1 ml enzyme solution. The mixture was incubated at 25°C for 15 minutes. The reaction was stopped by adding 1 drop of conc. HCl, and the abosrbance read at 402 nm. A standard curve was constructed using varying concentrations of hydrogen peroxide (0-3.0 µg/ml) in place of enzyme solution (Fig. 4). One enzyme unit was defined as that amount of enzyme which catalyzes the production of 10^{-3} µmole H_2O_2 per minute at 25°C, pH 6.3, and substrate concentration of 0.05 M.

Fig. 4. Standard curve for determination of units of enzyme activity.



Chlorella assay:

Assays with Chlorella pyrenoidosa (UNH strain) were done in buffered agar plates as previously described (1). To a Chlorella-seeded plate was added a ¼" sterile paper disk (Difco) which contained approximately 20 µl of test solution. After several days exposure to continuous fluorescent lighting, zones of inhibition appeared as colorless areas against a green background. The zone diameter minus the disk diameter was referred to as the 'net inhibition zone'.

Collection, Drying, and Grinding of Chondrus crispus

Chondrus crispus was collected year-round in the inter-tidal zone at Rye Beach, New Hampshire. Freshly collected fronds were taken to the laboratory as soon as possible where they were washed with cold tap water, blotted, and allowed to air-dry at room temperature for several days. Air-dried fronds were ground to a powder (#16 mesh) with a Wiley mill and then stored in a freezer prior to extraction.

Extraction of Chondrus crispus

To a 100 g sample of air-dried ground C. crispus fronds was added 1000 ml of 0.1 M sodium phosphate buffer pH 6.8. The mixture was kept at 5°C for 1-2 days during which time it was shaken periodically by hand. The mixture was then filtered through cheesecloth using gentle suction and the filtrate with washings were collected in an

ice-cooled flask. The residue which still contained some activity was discarded. The extract was further clarified by centrifugation at 20,000 x g for 30 minutes. The bright, red-orange supernate was recovered and purified by the following procedure.

Purification of the C. crispus Enzyme

All steps during purification were carried out at 0-5°C unless stated otherwise.

Step 1. n-Butanol extraction. The 20,000 x g supernate was mixed with an equal volume of n-butanol and after standing for several minutes, the mixture was centrifuged at 10,000 x g for 30 minutes, This treatment, as described by Leibo and Jones (19), caused a deposition of the unwanted photosynthetic pigment phycocyanin at the interface. The aqueous phase, red-orange in color due to the presence of phycoerythrin, was removed and the butanol fraction discarded.

Step 2. Ammonium sulfate precipitation. To the butanol-treated extract was slowly added with shaking, solid ammonium sulfate at 45 g/100 ml. After standing for several hours, the contents were centrifuged at 12,000 x g for 20 minutes. The precipitate was dissolved with stirring in 50-100 ml of 0.01 M sodium phosphate buffer pH 6.8. This solution was transferred to dialysis tubing and dialyzed against a minimum of four 2-liter changes of distilled water over a period of 2-3 days. Insoluble material in the retentate was removed by centrifugation at

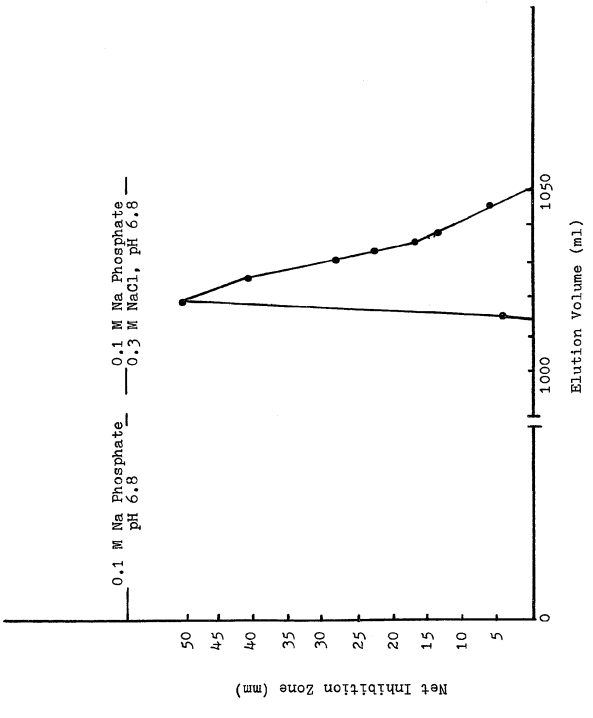
10,000 x g for 10 minutes. To the supernate was added sodium phosphate sufficient to make the solution 0.1 M pH 6.8.

Step 3. DEAE-cellulose chromatography. A DEAE-cellulose column (1.5 x 12 cm) was prepared using 10 g of Whatman DE 52 ion exchange cellulose and equilibrated with 0.1 M sodium phosphate pH 6.8. The sample which had been equilibrated with the same buffer was applied to the column. Following sample application, the column was washed with 500 ml of the same buffer used for equilibration. Stepwise addition of this buffer containing 0.3 M NaCl resulted in desorption of the Chondrus enzyme from the column. Fractions from the DE 52 column (Fig. 5) showing activity in the Chlorella assay were pooled and dialyzed against several 1-liter changes of distilled water overnight.

Step 4. Pepsin-trypsin treatment. The retentate was adjusted to pH 3.5 with dilute HCl (final volume ca. 80 ml). To the acidified solution was added 20 mg pepsin (3X crystallized) and the mixture incubated with shaking for 5 hours at 37°C. The reaction was stopped by adjusting the pH to 6.8 with dilute NaOH. Sodium phosphate was added to the digest to make the solution 0.01 M pH 6.8 with respect to phosphate. The mixture was then treated with 20 mg trypsin (2X crystallized) with shaking for 5 hours at 37°C. Following this treatment, the digest was freeze-dried.

Step 5. Gel filtration. The lyophilized digest

Fig. 5. DEAE-cellulose chromatography of *Chondrus* hexose oxidase.



was suspended in 3 ml of distilled water and applied to a column (2.5 x 96 cm) of Sephadex G-200 and the column developed with 0.1 M sodium phosphate pH 6.8. Fractions showing activity in the Chlorella assay (Fig. 6) were pooled, dialyzed extensively against distilled water and freeze-dried.

Fig. 6. Gel filtration of Chondrus hexose oxidase on Sephadex G-200. Vo=void volume determined with Blue Dextran (MW 2 x 10^6).

RESULTS

Initial Studies on The Growth-Inhibitory Substance in Chondrus crispus: Clues to Its Nature and Mode of Action

The effect of Chondrus crispus on Chlorella pyrenoidosa is shown in Fig. 7. Identical results are found with Euthora cristata. The effect of inhibition appears as a colorless area against a green background. Other algae which have also been found to inhibit the growth of C. pyrenoidosa (UNH strain) although not as greatly are Polysiphonia nigrescens and Membranoptera alata. Algae which are not inhibitory include Gigartina stellata, Sacchoriza dermatodea, Polysiphonia nigra, P. elongata, P. fibrillosa, P. urceolata, Ahnfeltia plicata, Ceramium strictum, and Lomentaria orcadensis. Whether P. nigrescens and/or M. alata contain a growth-inhibitory substance similar to that found in C. crispus and E. cristata is not known.

Some of the properties obtained for the active principle in C. crispus at the beginning of this investigation were the following: heat labile, sensitive to extreme pH, resistant to pepsin and trypsin, resistant to DNase and RNase, and high molecular weight (non-dialyzable) which were somewhat suggestive of a protein. However, the large zones of inhibition were difficult to explain in terms of diffusion of such a large substance as a protein. For this reason, the possibility of an enzymatic reaction

Fig. 7. Effect of the red alga Chondrus crispus on Chlorella pyrenoidosa (UNH strain).

which resulted in a toxic end-product low enough in molecular weight to account for large diffusion zones was considered. An experiment designed to test this hypothesis involved placing both ground C. crispus fronds and also a dialyzed aqueous extract, each contained in dialysis tubing, on Chlorella-seeded agar plates. Large zones of inhibition were found in both instances which indicated the diffusion of a substance in the Chlorella medium into the dialysis tubing where a reaction occurred that resulted in the production of a diffusible toxic substance. Of the ingredients present in the Chlorella medium (1), the most likely compound from which a toxic product could arise was D-glucose. The enzyme best known to oxidize D-glucose to an acid and hydrogen peroxide was glucose oxidase. The production of H_2O_2 by the action of a similar enzyme in Chondrus was thus indeed possible. An additional clue to the identity of the growth-inhibitory substance was obtained by adding an excess of catalase to a sterile paper disk ($\frac{1}{4}$", Difco) which also contained an extract of C. crispus. In the absence of catalase, inhibition was found while the catalase-treated sample showed no inhibition, thus providing the first direct evidence for the involvement of H_2O_2 in the growth-inhibitory response.

Evidence for Algal Origin of Hexose Oxidase in C. crispus

Several genera of marine bacteria have been isolated from the alga Porphyra leucosticta (20) and it

is possible that in this alga or other red algae such bacteria may contain enzymes having "glucose oxidase" activity. In order to establish the algal origin for the hexose oxidase, experiments were conducted to eliminate the possibility of microbial contamination of C. crispus as being the enzyme source. Finely ground samples of C. crispus and Euthora cristata as well as sodium phosphate buffered extracts were screened for such contaminating microbes. Growth studies were done using 2216 E medium, a modification of ZoBell's 2216 medium (21), which consisted of 0.1% peptone (Difco), 0.1% yeast extract (Fisher), 1.5% agar (Difco), and 0.001% ferric ammonium citrate made to 1 liter with 75% sea water (Seven Seas Marine Mix, Utility Chemical) and adjusted to a pH between 7.6 and 7.8 with 1.0 N NaOH. Microbial growth resulting after several days both on solid and liquid (agar omitted) media at 5, 18, and 25°C was collected and plated in excess directly on a Chlorella-seeded plate. No growth-inhibitory activity was found associated with the colonies isolated from solid media or pellets from centrifuged liquid culture which indicates the hexose oxidase found in C. crispus is of algal origin.

Purification of Chondrus hexose oxidase

Chondrus hexose oxidase was purified 117-fold with a recovery of 11% of the original activity (Table I). Approximately 10 mg of purified enzyme were obtained from

Table I

Purification of hexose oxidase from Chondrus crispus

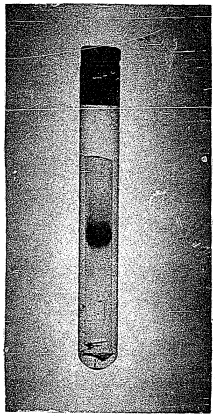
Stage of Purification	Volume (ml)	Total protein (mg)	Total carbohydrate (mg)	Total activity (Units)	Specific activity (Units/mg protein)	Yield (%)
20,000 x g supernatant	690	2,277	5,520	81,420	35	100
Ammonium sulfate ppt	425	468	527	69,700	149	85
DEAE-cellulose	84	76	29	49,340	650	66
Sephadex G-200	59	2	7	8,190	4,095	11

100 g of air-dried fronds. Disc gel electrophoresis of the purified enzyme showed a single band staining with Coomassie blue (Fig. 8). The Chondrus enzyme appeared unaffected by pepsin-trypsin digestion as shown by no loss in biological activity with the Chlorella assay and no alteration in molecular size when examined by gel filtration on Sephadex G-200. The digestion step was necessary to remove the red pigment phycoerythrin which persisted as an impurity in preparations of the enzyme. The bulk of this pigment however was removed by DEAE-cellulose chromatography since it was not adsorbed in the presence of 0.1 M sodium phosphate pH 6.8 and hence washed through the column.

Composition and molecular weight of Chondrus hexose oxidase

The enzyme showed a carbohydrate content of approximately 70% by the anthrone method using D-galactose as standard and 20% protein by the Lowry method based on bovine serum albumin. Moisture may account for ca. 10% of the weight of the lyophilized enzyme. The carbohydrate composition of Chondrus hexose oxidase was determined on a 1 mg sample of enzyme which was hydrolyzed with 1 ml of 2 N H_2SO_4 for 4 hours in a boiling water bath. Solid $BaCO_3$ was added to the hydrolysate until the pH was approximately 5 and the mixture was then centrifuged. The supernatant and washings from the $BaSO_4$ precipitate were combined and concentrated, and the concentrate was chromatographed along with known sugars on Whatman No. 1 paper in the following

Fig. 8. Disc gel electrophoresis of purified Chondrus hexose oxidase.



systems: n-butanol:ethanol:water (2:1:1, v/v) (15), benzene:n-butanol:pyridine:water (1:5:3:3, v/v) (15), n-butanol:pyridine:water (45:25:40, v/v) (22), and ethyl acetate:pyridine:water (2:1:2, v/v) (22). The chromatograms were sprayed with either aniline hydrogen phthalate or aniline hydrogen oxalate (22). Galactose and xylose were identified as the principal sugars in the Chondrus enzyme. Galactose appeared to be the predominant sugar, because, based on a galactose standard, the carbohydrate content of the enzyme was estimated at 70%, whereas, based on a xylose standard, the carbohydrate content calculated out as 115%, due to a lower color yield from xylose. The amino acid composition was determined with a Spinco Amino Acid Analyzer on a 4.7 mg sample of enzyme which had been hydrolyzed in 6 N HCl at 110°C for 24 hours (Table II). It appeared rich in aspartic acid, threonine, serine, glutamic acid, glycine, alanine, and valine, and low in the basic amino acids (lysine, histidine, and arginine, the sulfur-containing amino acids (cysteine, methionine) and the aromatic amino acids (tyrosine, phenylalanine). Tryptophan was not determined. Without corrections for loss or degradation, the total weight of amino acids was calculated from the analysis to be 605 ug or ca. 13% of the sample weight which showed agreement with the low value from the Lowry determination.

The glycoprotein nature of Chondrus hexose oxidase was further demonstrated by staining with Alcian Blue

Table II

Amino acid composition of Chondrus hexose oxidase

Amino acid	μMole/mg enzyme	Molar ratio*
Lysine	0.0447	5
Histidine	0.0083	1
Ammonia	----	-
Arginine	0.0247	3
Aspartic acid	0.1689	20
Threonine	0.0851	10
Serine	0.1223	14
Glutamic acid	0.1647	20
Proline	0.0723	9
Glycine	0.1483	18
Alanine	0.1140	14
Half-cystine	0.0264	3
Valine	0.0832	10
Methionine	0.0179	2
Isoleucine	0.0357	4
Leucine	0.0621	8
Tyrosine	0.0198	2
Phenylalanine	0.0459	6
Tryptophan	----	-

*Obtained by normalizing values relative to histidine = 1.

following cellulose acetate elcetrophoresis (23). By this procedure, both the Chondrus enzyme and glucose oxidase showed a blue band against a pale blue background. Sections from an unstained cellulose acetate strip coinciding with the stained band were excised and placed in the o-dianisidine-peroxidase mixture (see MATERIALS AND METHODS). The rapid formation of a yellow-orange color indicated the association of "glucose oxidase" activity with the band stained for glycoprotein. Staining a developed strip containing Chondrus enzyme with Ponceau S (24) resulted in a pink-red band against a pink background having the same mobility as those sections having enzyme activity and staining with Alcian Blue. Chondrus hexose oxidase failed to stain with Schiff's reagent (24) which is not uncommon for glycoproteins rich in carbohydrate (23).

An emission spectrum of the enzyme showed copper (with a trace of sodium) to be the only metal present. Using the dithizone method and atomic absorption spectroscopy, a value of approximately 0.6% (6 μg/mg enzyme) was obtained. For example, a 9.8 mg sample showed total copper by the dithizone method to be 66 μg and by atomic absorption to be 58 μg. Slight variation was found between determinations for two individually processed samples of purified enzyme (by the dithizone method 0.54-0.67% Cu). Both methods appeared relatively close in agreement.

Qualitative determination of flavin adenine dinu-

cleotide (FAD) was done by the method of Pazur and Kleppe (8) which involved treatment of the enzyme at 45°C for 15 minutes with pyridine. Under such conditions the flavin group of glucose oxidase dissociated. This result was confirmed using glucose oxidase and the split FAD examined by paper chromatography. Using Whatman No. 1 paper with a solvent system consisting of n-butanol:acetone:acetic acid: water (5:2:1:3, v/v) which has been described by Pazur and Kleppe (8), the following R_f values were obtained after exposing the developed chromatograms to ultraviolet light: FAD = 0.10, FMN = 0.24, and treated glucose oxidase = 0.10. (FMN = flavin mononucleotide) A lyophilized 2 mg sample of Chondrus enzyme (pale green in color), treated similarly, showed no trace of FAD. The same result was found with a 5-10 minute treatment in a boiling water bath. The treated Chondrus enzyme in both instances showed no fluorescence unlike denatured glucose oxidase and flavin standards. Supplemental evidence for the absence of FAD was based on a rather featureless visible spectrum which unlike glucose oxidase showed no discernable peaks even at 380 and 460 nm (25) which are characteristic of FAD (Fig. 9).

An approximate molecular weight was obtained by gel filtration on Sephadex G-200. A column (2.5 x 43 cm) was equilibrated at 5°C with 0.1 M sodium phosphate pH 6.8 and several proteins of known molecular weight were used as standards (Fig. 10). The elution volume for the Chondrus enzyme corresponded to a molecular weight of

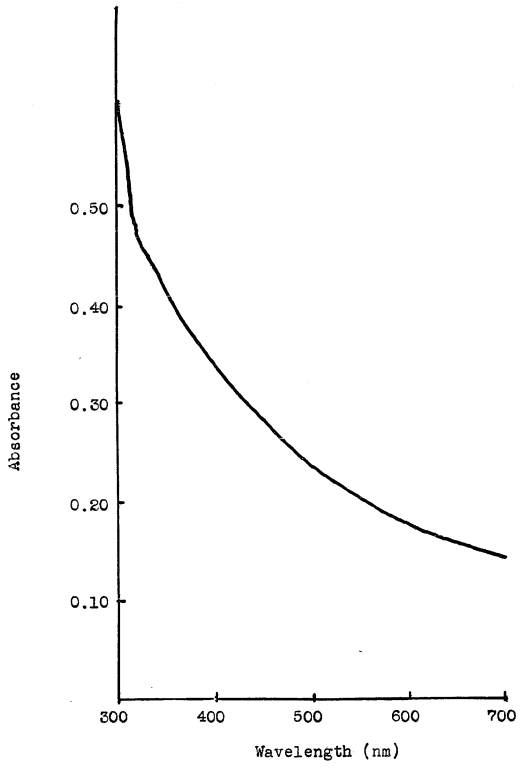
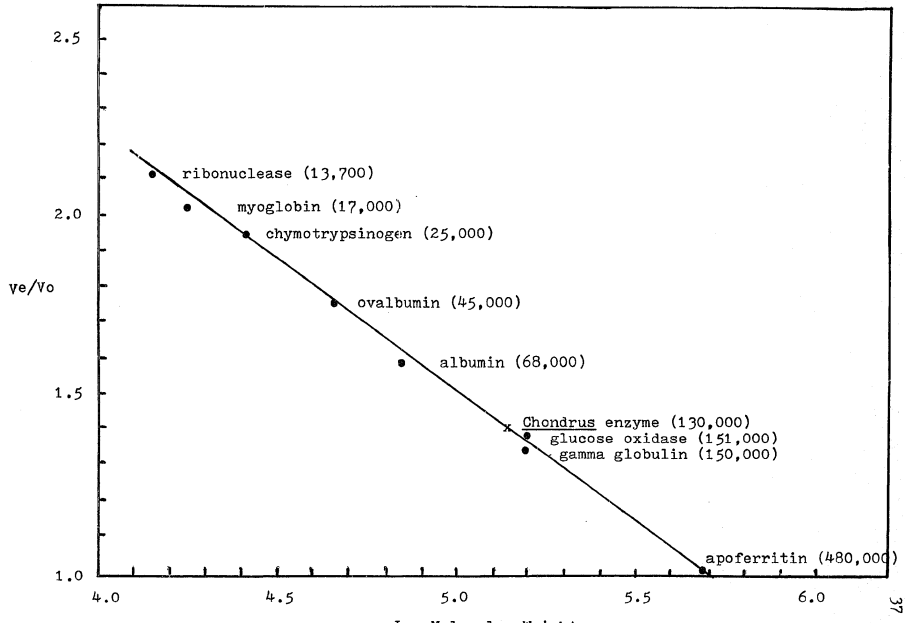


Fig. 9. Visible spectrum of purified Chondrus enzyme. Sample was ca. 5 mg/ml in distilled water.

Fig. 10. Relationship of elution volume to molecular weight for several protein standards and Chondrus hexose oxidase on a column of Sephadex G-200 (2.5 x 43 cm).



ca. 130,000.

Properties of Chondrus hexose oxidase

The pH optimum of the enzyme was determined using 0.1 M sodium phosphate buffers ranging in pH from 4.9 to 9.1. The optimum pH appeared to be ca. 6.3 (Fig. 11). The enzyme was found most active at an incubation temperature of 25°C (Fig. 12). Heat stability of the enzyme was determined by heating for 5 minutes at various temperatures, chilling in an ice bath, and assaying with the o-dianisidine-peroxidase system (Fig. 13). A sudden drop in activity occurred between 50 and 60°C.

Substrate specificity of the enzyme was determined using a number of sugars at a final concentration of 0.1 M (Table III). The substrates most readily oxidized were D-glucose, D-galactose, maltose, cellobiose, and lactose. L-glucose was not oxidized. The five main substrates of the Chondrus enzyme, at a final concentration of 0.1 M, were tested with a partially purified extract of Euthora cristata and also with glucose oxidase (Table IV). The Euthora preparation gave essentially the same results as the Chondrus enzyme but glucose oxidase attacked only D-glucose at a significant rate. In order to determine whether free glucose might be present in the disaccharide samples, 1% solutions of each sugar were chromatographed on Whatman No. 1 paper in either ethyl acetate:pyridine: water (120:50:40, v/v) or iso-propanol:water (4:1,v/v) and the chromatograms sprayed with aniline hydrogen phthalate

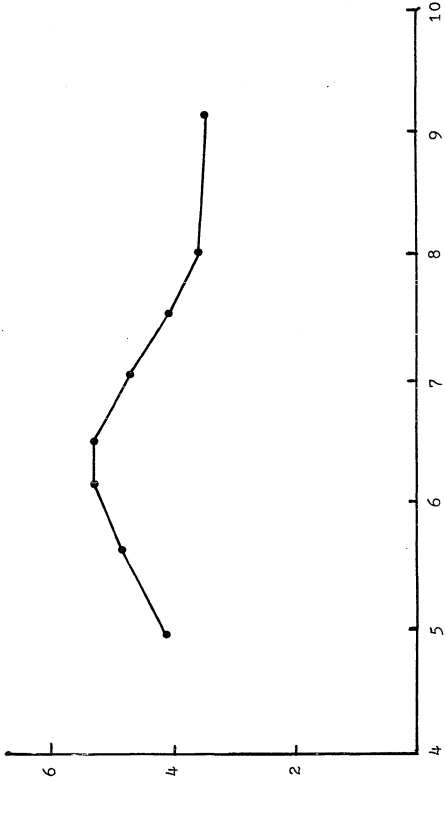


Fig. 11. Effect of pH on enzyme activity.

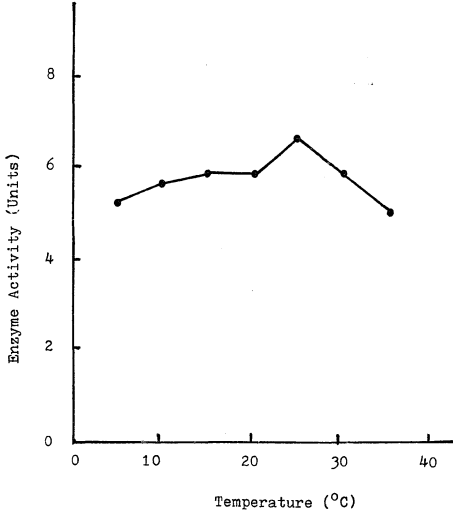


Fig. 12. Effect of incubation temperature on enzyme activity.

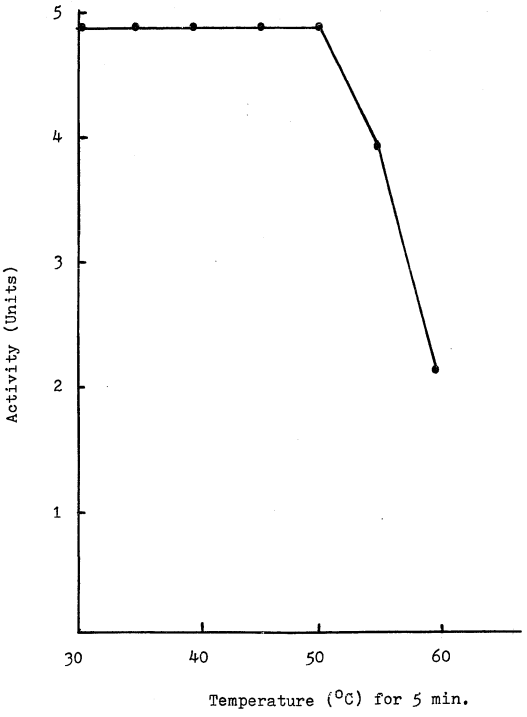


Fig. 13. Heat stability of Chondrus hexose oxidase.

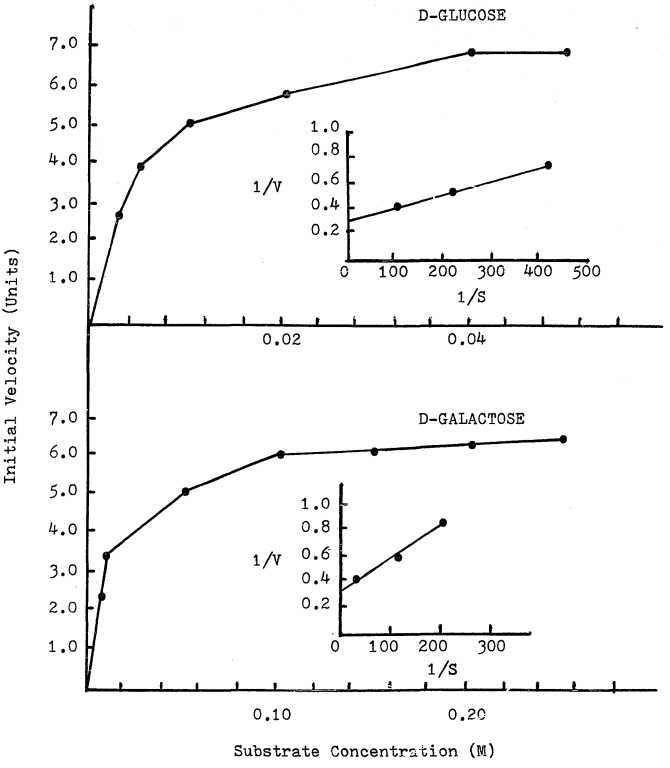


Fig. 14. Effect of substrate concentration on reaction velocity.

Table III

Substrate specificity of Chondrus hexose oxidase

Substrate*	Relative Rate
D-Glucose	100
D-Galactose	82
Maltose	40
Cellobiose	32
Lactose	22
Glucose 6-phosphate	10
D-Mannose	8
2-Deoxy D-Glucose	8
2-Deoxy D-Galactose	6
D-Fucose	2
D-Glucuronic acid	2
D-Xylose	1

*Sugars not oxidized:

L-glucose, D-fructose, D-gluconic acid lactone, γ-galactonolactone, dulcitol, D-gluconic acid, D-arabinose, xylitol; sucrose.

Table IV

Comparison of substrate specificity of *Euthora* and *Chondrus* enzymes and glucose oxidase

	Relative Rate		
Substrate	*Euthora* enzyme*	*Chondrus* enzyme	Glucose Oxidase
D-Glucose	100	100	100
D-Galactose	95	82	0
Maltose	32	40	1
Cellobiose	95	32	2
Lactose	51	22	0

*Partially purified sample obtained from DEAE-cellulose column (see METHODS).

followed by heating at 100°C for 5 minutes. A trace of free glucose was detected only in the sample of maltose.

The effect of increasing substrate concentration on reaction velocity was determined with D-glucose and D-galactose (Fig. 14), and the method of Lineweaver-Burk (26) was used to determine Michaelis constants (Km's). The Km's for D-glucose and D-galactose were 0.004 M and 0.008 M, respectively. A Km of 0.0025 M for D-glucose was reported for the Iridophycus enzyme (3).

The effect of various inhibitors on the Chondrus enzyme was determined (Table V). The most potent inhibitor was sodium diethyldithiocarbamate, effective at 10^{-5} M. This compound also inhibited glucose oxidase at this level. The enzyme was also inhibited by sodium cyanide, sodium azide, hydroxylamine hydrochloride, sodium acetate, and sodium pyruvate. The Iridophycus enzyme was reported as being quite sensitive to acetate (3), more so than found with the Chondrus enzyme.

Products of Chondrus hexose oxidase

The production of hydrogen peroxide in the Chondrus enzyme reaction was shown by omitting peroxidase from the standard assay mixture. When this was done, o-dianisidine was very slowly oxidized to a colored product. Since peroxidase specifically uses H_2O_2 to oxidize the o-dianisidine, this demonstrates that H_2O_2 is being produced. Additional evidence was obtained by including an excess of catalase with Chondrus enzyme

Table V

Effect of various inhibitors on Chondrus hexose oxidase

Inhibitor*	Concentration (M)	Inhibition (%)
Sodium diethyl-dithiocarbamate	10^{-4}	95
	10^{-5}	22
Sodium cyanide	10^{-3}	61
	10^{-4}	15
Hydroxylamine hydrochloride	10^{-2}	100
	10^{-3}	96
	10^{-4}	26
Sodium azide	10^{-1}	85
	10^{-2}	78
	10^{-3}	65
Sodium acetate	10^{-1}	56
	10^{-2}	13
Sodium pyruvate	10^{-1}	43

*Showed no inhibition at 10^{-2} M: sodium pyruvate, sodium benzoate, D-gluconic acid, D-gluconic acid lactone and D-glucuronic acid.

using the Chlorella assay. In the presence of catalase, no inhibition of Chlorella was found. However, in the absence of catalase inhibition occurred. The H_2O_2 was decomposed by catalase to water and oxygen both of which are obviously non-toxic to Chlorella. The toxic effect of H_2O_2 to Chlorella was shown by testing this compound at various concentrations: a net zone of inhibition of 3.8 cm was found at 10 mg/ml, a 1.6 cm net zone at 1.0 mg/ml, and a net zone of 0.2 cm at 0.1 mg/ml. A paper disk treated with glucose oxidase showed inhibition when tested against Chlorella apparently due to the production of H_2O_2 since D-gluconolactone at 10 mg/ml was not inhibitory.

The product in addition to H_2O_2 formed by the Chondrus hexose oxidase reaction was determined by incubating the enzyme plus excess catalase in 2 ml of 0.1 M glucose in 0.1 M pH 6.3 sodium citrate buffer at 25°C for ca. 12 hours. Also reacted under the same conditions was glucose oxidase. Paper chromatography of the reaction mixtures after 12 hours or longer showed the formation of D-gluconolactone from D-glucose and D-galactonolactone from D-galactose (Table VI). The oxidation of D-glucose and D-galactose by Chondrus hexose oxidase can therefore be written as shown in the following reactions:

Table VI

Paper chromatography of products from the Chondrus hexose oxidase reaction

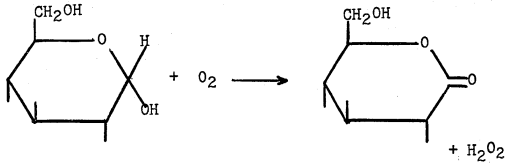
Sample*	R_f values †	
	Solvent A	Solvent B
Glucose	0.14	0.35
δ-D-Gluconolactone	0.37	0.54
Chondrus enzyme product from glucose	0.37	0.46
Glucose Oxidase product	0.37	0.46
Galactose	0.13	0.42
γ-D-Galactonolactone	0.32	0.46
Chondrus enzyme product from galactose	0.32	0.44

*Glucose and galactose were detected with aniline hydrogen phthalate spray (22) and the lactones and oxidation products by spraying with hydroxylamine and ferric chloride (29).

†Run on Whatman No. 1 paper. Solvent systems used by Bean et al. (11).

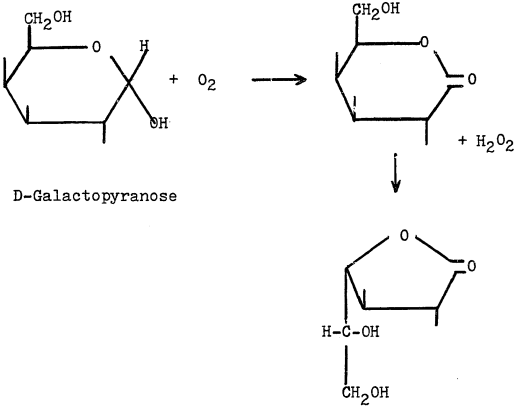
A = n-butanol:acetic acid: water (52:13:35).

B = Phenol:water (80:20).



D-Glucopyranose

δ -D-Gluconolactone



D-Galactopyranose

γ-D-Galactonolactone

DISCUSSION

The substance in Chondrus crispus which is responsible for inhibiting the growth of Chlorella pyrenoidosa has been shown to be hexose oxidase. This enzyme reacts with D-glucose in the Chlorella medium producing the oxidized sugar and hydrogen peroxide. Of the two products formed, H_2O_2 has been determined as the actual growth-inhibitory compound. Chondrus hexose oxidase has a wide substrate specificity which includes principally D-glucose, D-galactose, maltose, lactose, and cellobiose. D-glucose and D-galactose are oxidized by this enzyme to the corresponding hexonolactones. Quite similar in properties to the Chondrus enzyme is a hexose oxidase obtained from the red alga Iridophycus flaccidum (3). It likewise produces H_2O_2 and the aldonic acid (lactone) from D-hexoses (3). Another red alga, Euthora cristata, has been shown to contain a hexose oxidase and as with Chondrus its inhibition to Chlorella is due to H_2O_2.

In addition to hexose oxidase, various other "glucose oxidases" have been reported. Table VII presents a comparison of these enzymes, their sources, and some of their properties. The sources include red algae, citrus fruits, fungi, bacteria, and honey. The most studied of these enzymes has been glucose oxidase found in the fungi Aspergillus and Penicillium. This enzyme contains 2 FAD per molecular weight of 150,000 to 160,000. Lactose

Table VII

A comparison of properties for various "glucose oxidases"

Enzyme Source	Optimum pH	Optimum Temp (°C)	Coenzyme	Molecular Weight	Substrate(s)	Reference
Iridophycus flaccidum	5.0	30	—	—	gluc, gal, mal, lac; cell.	3
Polyporus obtusus	6-8	25	—	—	gluc, xyl, L-sorb, gluconolactone.	9
Citrus fruits	6.0	—	—	—	gluc, gal, man, 2-deoxy gluc, xyl, lac, cell, mal, 2-gluc amine.	11
Aspergillus niger	5.5	30	2 FAD	150,000	gluc	8
Penicillium amagasakiense	5.6	30	2 FAD	160,000	gluc	10
P. notatum	5.6	39	2 FAD	152,000	gluc	7
Honey	6.1	37	—	<100,000	gluc	4
Chondrus crispus	6.3	25	12 Cu	130,000	gluc, gal, mal, lac; cell.	(this thesis)
Euthora cristata	—	—	—	—	gluc, gal, mal, lac; cell.	(this thesis)
Malleomyces pseudomallei	5.5	—	—	—	gluc, gal	5
Pseudomonas graveolens	—	—	FAD	—	lac	6

dehydrogenase also contains FAD however its molecular weight has not been reported. Regarding substrate specificity, the honey enzyme and glucose oxidase are similar in that both are highly specific for D-glucose. FAD has not been detected in the honey enzyme and no requirement for it has been indicated (4).

A coenzyme other than FAD has not previously been reported for a "glucose oxidase". The finding of a copper-containing enzyme with glucose oxidase activity in Chondrus is new. Whether other "glucose oxidases" contain copper is not known. It is suspected however based on similarity in properties that the Iridophycus and Euthora enzymes also contain copper.

The Chondrus enzyme contains approximately 12 gram atoms of copper per mole and apparently no FAD. This large amount of copper can be contrasted to the 1 gram atom per mole reported for galactose oxidase (12). The two enzymes are related in that each contains copper and both produce H_2O_2 from oxidation of their substrates. They can be clearly distinguished, however, because substrate oxidation with galactose oxidase occurs at the C-6 position resulting in formation of a dialdehyde from D-galactose while C-1 oxidation is found with the Chondrus enzyme with the product being hexonolactone. Like glucose oxidase, galactose oxidase is quite substrate specific, however, the galactose enzyme also oxidizes galactose-containing polysaccharides quite well (13).

Besides containing a high level of copper, the Chondrus enzyme has been found to contain ca. 70% carbohydrate compared to 17% reported for glucose oxidase (27). The carbohydrate moiety of Chondrus hexose oxidase consists principally of galactose and xylose while glucose oxidase contains 14% mannose, 2% glucosamine, and 1% galactose (27). The importance of carbohydrate to the Chondrus enzyme's activity has not been determined. Treatment with periodic acid could resolve this point such that loss or reduction in activity would be due to destruction of the carbohydrate portion of the enzyme. The enzymatic activity of glucose oxidase, however, remains unaffected by mild periodate oxidation indicating the carbohydrate residues are probably not involved in the enzyme's active site (27).

The glycoprotein nature of Chondrus hexose oxidase has been shown by staining developed strips from cellulose acetate electrophoresis with Alcian Blue (23). A blue band against a pale blue background is shown by the Chondrus enzyme and glucose oxidase. Enzymatic activity in both cases is associated with sections of the strips stained with Alcian Blue. Staining a developed strip containing Chondrus enzyme for protein with Ponceau S (24) gives a pink-red band against a pale pink background which is identical in mobility to those sections staining with Alcian Blue and showing enzyme activity.

Chondrus hexose oxidase appears to be a rather

stable enzyme such that losses in activity do not occur following pepsin-trypsin digestion, heating at 50°C for 5 minutes, or extensive dialysis against distilled water. Its stability to proteolytic digestion can be explained in part due to its amino acid composition which reflects low levels of tyrosine, phenylalanine, lysine, and arginine. Pepsin would be expected to preferentially hydrolyze at sites adjacent to aromatic amino acids while trypsin would favor cleavage adjacent to lysine or arginine. Perhaps more important than the actual number of these amino acids would be their arrangement or position in the overall structure such that they would or would not be in an accessible location for proteolytic attack. Resistance to pepsin-trypsin digestion is also found with glucose oxidase (28). The Chondrus enzyme's stability to dialysis against distilled water suggests that the copper is tightly bound to the enzyme. This same type of stability to extensive dialysis is also shown by galactose oxidase (12).

The purified Chondrus enzyme is pale green in color which probably results from its high copper content. The lyophilized enzyme was on occasion difficult to handle because of its very hygroscopic nature. Within a minute after being disconnected from a lyophilizer on a humid day, it changes to a sticky material with a greenish color. This rapid hydration may be related to the large amount of bound copper and possibly the carbohydrate content.

A molecular weight of approximately 130,000 is

shown by Chondrus hexose oxidase on a column of Sephadex G-200 which had been calibrated with proteins of known molecular weight. This value could differ from the actual molecular weight by 10% or more. For example, glucose oxidase is slightly retarded on this column and hence shows a molecular weight lower than the actual value. Gel filtration appears to provide at least an estimate of the actual molecular weight. Determination of molecular weight by additional methods will be necessary to confirm this value.

A number of compounds have been found to inhibit the Chondrus enzyme, most severely being sodium diethyldithiocarbamate effective at 10^{-5} M. In decreasing order of effectiveness are sodium cyanide, hydroxylamine hydrochloride, sodium azide, sodium acetate, and sodium pyruvate, Diethyldithiocarbamate at 10^{-4} M has been reported to inhibit completely the enzymatic action of galactose oxidase (12). Also sensitive to this inhibitor to the same extent is glucose oxidase.

SUMMARY AND CONCLUSIONS

The marine red alga Chondrus crispus has been found to contain a "glucose oxidase" which is responsible for the observed inhibition to Chlorella pyrenoidosa through its action on glucose in the Chlorella medium which results in the production of the growth-inhibitory compound hydrogen peroxide. An 11% recovery of activity is realized from a purification procedure involving extraction with 0.1 M sodium phosphate pH 6.8, n-butanol treatment, DEAE-cellulose chromatography, pepsin-trypsin digestion, and gel filtration on Sephadex G-200. The enzyme appears unique in having "glucose oxidase" activity while lacking FAD and containing copper instead. Preliminary studies on Euthora cristata indicate it also contains a hexose oxidase.

There are additional experiments which would surely provide a better understanding of the Chondrus enzyme. Such studies as determining the relevance, if any, of the carbohydrate portion to activity, the manner in which the copper is coordinated in the native enzyme, further work on the subunit composition, and molecular weight are but a few examples.

Chondrus crispus is found in abundance at Rye Beach, New Hampshire in the inter-tidal zone. Adequate supply of this alga should therefore not present a problem for future study. Euthora cristata however has

been collected only from the drift and supply has been rather limited. As this alga grows in deeper water the only way by which sufficient material could be collected would be by diving.

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APPENDIX

During the course of this work a number of compounds have been tested against strains of Chlorella. The results of these tests are presented in the following tables.

Table IA which has been taken directly from a paper by Sullivan and Ikawa (2) shows the activity of some toxins and inhibitors on Chlorella strains. It is apparent from these results that strains of Chlorella differ in sensitivity to certain compounds. Perhaps the most sensitive strains are UNH and 395 of C. pyrenoidosa. For a more lengthy discussion of this material the reader is referred to the original paper (2).

The effect of several pesticides on Chlorella strains is shown in Table IIA. As found in Table IA, there exists here also a variation in sensitivity among Chlorella strains in this case to pesticides. C. pyrenoidosa (UNH strain) and C. vulgaris are not inhibited by these pesticides when tested at 1 mg/ml.

In Table IIIA are found the results of testing a variety of miscellaneous compounds and antibiotics against C. pyrenoidosa (UNH strain).

A number of steroidal compounds have been also tested although none have proven inhibitory to Chlorella (Table IVA).

Table IA

Growth-Inhibitory Activity of Some Toxins and Inhibitors on *Chlorella* Strains[a]

Compound	Solvent	Conc. (mg/ml)	Diameter of Net Zone of Inhibition, mm[b]				
			C. pyrenoidosa				
			UNH	395	251	252	*C. vulgaris*
Acrylic acid	Ethanol	1	36	35	0	0	0
	Ethanol	0.1	15[c]	0			
	Ethanol	0.01	0				
Aflatoxin B_1	DMSO[d]	1	25	18	21	21	0
	DMSO	0.1	0	0	0	0	
Aflatoxin B_2	DMSO	1	0	0	0	0	0
Aflatoxin G_1	DMSO	1	0	0	0	0	0
Aflatoxin G_2	DMSO	1	0	0	0	0	0
Aflatoxin B_1A[e]	DMSO	1	0	0	0	0	0
Diacetoxy-scirpenol	Ethanol	1	40[c]	39[c]	8[c]	8[c]	4
	Ethanol	0.1	19[c]	26[c]			
	Ethanol	0.01	Tr[f]	Tr			
Digitonin	Ethanol	1	17	25	0	0	20[c]
Emodin	Ethanol	1	4	6	6	6	Tr
Gramicidin J	Water	1	10	13	0	0	28
	Water	0.1	Tr	Tr			14
	Water	0.01					Tr

Table IA (continued)

Compound	Solvent	Conc. (mg/ml)	Diameter of Net Zone of Inhibition, mm[b]				
			C. pyrenoidosa				
			UNH	395	251	252	C. vulgaris
Kainic acid	Ethanol	1	0	0	0	0	0
Rubratoxin B	Ethanol	1	0	0	0	0	0
Zearalenone[g] (F-2)	Ethanol	1	15[c]	Tr	14[c]	13[c]	0
	Ethanol	0.1	0	0	0	0	

[a]Sterile disks (Difco Laboratories) of 0.6-cm diameter were used on buffered agar plates. Values of inhibition represent an average of at least three separate determinations each run in duplicate with two disks per plate. To illustrate the variability of response, the mean and standard deviation from the mean in the case of C. pyrenoidosa (UNH strain), on which the most assays were run, were 36 ± 1 for gramicidin J, and 15 ± 1 for zearalenone, when all compounds were tested at 1 mg/ml. [b]Diameter of disk subtracted from total diameter of inhibition zone. [c]Weak growth of Chlorella was observed within the inhibition zone which was surrounded by a background of denser growth. This often made reading the zones difficult. In the case of diacetoxyscirpenol, small zones at complete inhibition were also observed within the zone where partial growth had occurred. [d]DMSO = dimethylsulfoxide. A zone of weak Chlorella growth extending 3.5 mm beyond the disk was observed with DMSO as solvent. This zone had been ignored in previous work (Ikawa et al., 1969). [e]A synthetic analog of aflatoxin B_1 was kindly supplied by J.V. Rodricks, Food and Drug Administration, Washington, D.C. [f]Trace indicates a net zone of 3 mm or less. [g]F-2 crystals were kindly supplied by C.J. Mirocha, University of Minnesota, St. Paul, Minn.

Table IIA

Effect of Several Pesticides on *Chlorella* Strains

Compound	Conc. (mg/ml) *	Diameter of Net Zone of Inhibition, mm		
		UNH	*C. pyrenoidosa* Carolina 15-2070	*C. vulgaris*
Endrin	1	0	3	0
DDT	1	0	2	0
Dieldrin	1	0	4	0
Toxaphene	1	0	4	0
Aldrin	1	0	2	0
Lindane	1	0	6	0
	0.1		0	
Chlordane	1	0	3	0
Methoxychlor	1	0	6	0
	0.1		3	
Sevin	1	0	2	0

*Solvent = 95% ethanol

Table IIIA

Effect of Various Compounds on *Chlorella pyrenoidosa* (UNH strain)

Compound	Solvent	Conc. (mg/ml)	Diameter of Net Zone of Inhibition, mm
Rotenone	Ethanol	1	2
p-Benzoquinone	Ethanol	1	3
Oligomycin	Ethanol	1	6
Amobarbital	Ethanol	1	0
Antimycin A	Ethanol	1	7
Monsensin	Ethanol	1	0
Valinomycin	Ethanol	1	0
Streptomycin sulfate	Water	1	5
Achromycin • HCl	Water	1	0
Albamycin acid Na	Water	1	0
Penicillin G, K salt	Water	1	0
Chloramphenicol	Water	1	0
Bacitracin	Water	1	0
Coumarin	Ethanol	1	0
4-Hydroxy coumarin	Ethanol	1	0
Dicumarol	DMSO	1	0

Table IVA

Effect of Steroidal Compounds on Chlorella pyrenoidosa *

Compound	Diameter of Net Inhibition Zone, mm
8,24, 5α-cholestadien-4,4,14α-trimethyl 3β-ol	0
4α-cholesta-3-one	0
5α-cholestan-3-one	0
Stigmasterol	0
Progesterone	0
Testosterone	0
Pregnenolone	0
Deoxycorticosterone	0
Sitosterol	0
Ergosterol	0
Betulin	0
Hydrocortisone acetate	0
Androsterone	0
Cortisone acetate	0
Cholesterol	0
17β-estradiol	0
Estriol	0
Ouabain	0

* (UNH strain)

Compounds tested at 1 mg/ml in ethanol.